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Standard of hygiene and immune adaptation in newborn infants

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Abbreviations: APC, antigen presenting cell; DC, dendritic cell; HLA, human leukocyte antigen; LPS, lipopolysaccharide; TCR, T cell receptor; Th1, T helper cell type 1; Th2, T helper cell type 2; TLR, toll-like receptor; Treg, T regulatory cell.

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Abstract The prevalence of immune-mediated diseases, such as allergies and type 1 diabetes, is on the rise in the developed world. In order to explore differences in the gene expression patterns induced in utero in infants born in contrasting standards of living and hygiene, we collected umbilical cord blood RNA samples from infants born in Finland (modern society), Estonia (rapidly developing society) and the Republic of Karelia, Russia (poor economic conditions). The whole blood transcriptome of Finnish and Estonian neonates differed from their Karelian counterparts, suggesting exposure to toll-like receptor (TLR) ligands and a more matured immune response in infants born in Karelia. These results further support the concept of a conspicuous plasticity in the developing immune system: the environmental factors that play a role in the susceptibility/protection towards immune-mediated diseases begin to shape the neonatal immunity already in utero and direct the maturation in accordance with the surrounding microbial milieu.

1. Introduction

The prevalence of immune-mediated diseases in developed countries is continuously increasing and is strongly correlated with the standard of living. These observations have led to the formulation of the hygiene hypothesis [1,2], according to which environmental factors, such as microbial and parasite infections, encountered during early childhood suppress the development of harmful immune responses later in life. Lack of normal microbial contacts in the industrialized world could then predispose individuals towards allergies and autoimmune diseases. The education of the immune system takes place in early childhood, and at least partly begins already during pregnancy. As an example indicating prenatal Th2 cell priming and activity, allergen-specific IgE antibodies can be detected at the time of birth [3]. Environmental factors encountered in utero, such as exposure to farms, especially stables and hay [4], and maternal and grandmaternal smoking [5] are known to modulate atopy and asthma in the offspring.

Estonia, Finland and Russian Karelia are adjacent areas exposed to the same seasonal changes but differing in their socio-economic status (Fig. 1). Finland is the most modernized of these societies, Estonia, since gaining independence, has

Figure 1 Map of the three study sites located in Espoo, Finland; Tartu, Estonia; and Petrozavodsk, Russia.

experienced a rapid economic growth, and Russian Karelia has the poorest economic conditions of the three. In 2009, the gestational period covered by this study, the gross domestic product (in purchasing power parity, PPP) was 12,931 USD in the Republic of Karelia [6] and 19,451 USD in Estonia, as opposed to 34,720 USD in Finland [7]. This is further reflected by the life expectancy at birth, which in 2009 was 66.6 years in the Republic of Karelia [6], 74.3 years in Estonia, and 79.6 years in Finland [8]. Previous studies comparing Finnish school children to their peers in Russian Karelia have shown that the incidence of type 1 diabetes is almost six times higher in Finland [9]. In Estonia the incidence of type 1 diabetes is increasing and is currently more than two times higher than in Russian Karelia, and almost three times lower than in Finland [10,11]. The prevalence of asthma, hay-fever and allergies is also rising in Finland, but has remained stable in Russia [12]. Conversely, compared to Finnish children, Karelian children are more frequently exposed to microbial infections, such as *Toxoplasma gondii*, *Helicobacter pylori*, as well as Hepatitis virus A and Coxsackievirus B4, as measured by antimicrobial antibodies [13].

In order to evaluate the influence of these contrasting standards of living and infections, we have studied the similarities and differences in the gene expression patterns induced in utero in infants born in these adjacent countries. Whole blood RNA expression profiles from umbilical cord blood RNA samples, collected from infants born in Finland, Estonia and Russian Karelia (Fig. 1,Table 1) were analyzed with microarrays. Clear differences were observed in between the study sites, with Russian Karelia deviating most clearly from the Estonian and Finnish samples. A salient example was observed in the expression of transcripts associated with lipopolysaccharide (LPS) induction and sepsis, which were higher in the samples from Karelian neonates, suggesting in utero exposure to toll-like receptor (TLR) ligands. This provides a strong indication, that there is an in utero influence on susceptibility/protection towards immune-mediated diseases, which begins to shape the neonatal immunity and direct the maturation of both the adaptive and innate immune responses in accordance with the surrounding microbial milieu.

2. Subjects and methods

2.1. Subjects and sample collection

The samples were collected as part of the international DIABIMMUNE study, aimed at testing the hygiene hypothesis in the development of autoimmune and allergic diseases. Umbilical cord blood was drawn into Tempus Blood RNA tubes (Applied Biosystems) from children born between January and May 2010 at the maternity unit of Jorvi Hospital (Espoo, Finland; n = 48), maternity units of Tartu and Põlva (Estonia; n = 25), or two maternity departments in Petrozavodsk (capital of the Republic of Karelia, Russian Federation; n = 40) according to the manufacturer's protocol and then stored at −70 °C until analyzed. All newborn infants were full-term (>36 gestational weeks) and born vaginally (Tables 1 and S1). The HLA-DR-DQ genotypes related to

Table 1 Characteristics of the umbilical cord blood RNA samples collected at the three study sites. See Table S1 for further details on the samples.

	Espoo	Tartu	Petrozavodsk
Number of samples	48	25	40
Males/females	26/22	10/15	22/18
Average gestational week	39.3 (36–42)	39.4 (37–42)	39.6 (37–41)
HLA risk class [a]	NP: 36 samples	NP: 22 samples	NP: 34 samples
	M: 4 samples	M: 1 samples	M: 2 samples
	H: 6 samples	H: 1 sample	H: 4 samples
	VH: 2 samples	VH: 1 sample	VH: 0 samples
HLA-DR3-DQ2 positive [b]	8 samples	5 samples	4 samples
HLA-DR4-DQ8 positive [c]	12 samples	2 samples	7 samples
Birth dates	January–May 2010	January–May 2010	January–May 2010

[a] NP = neutral or protective genotypes, M = moderate risk associated genotypes, H = high risk associated genotypes, VH = very high risk associated genotypes (Peet et al. [14]).
[b] HLA-DQA1*05-DQB1*02 haplotype.
[c] HLA-DRB1*04:01/2/4/5/8-DQB1*0302/4 haplotype.

Table 2 Top 15 enriched canonical pathways among the differentially regulated genes, as reported by the Ingenuity Pathway Analysis (IPA) core analysis tool. The Genes/pathway column reports the number of differentially regulated genes in the comparison / total number of genes annotated to the pathway. The coloring of the rows is used to highlight the overlapping pathways observed in the three comparisons.

Espoo vs. Petrozavodsk		
Ingenuity canonical pathway	Genes/pathway	P value
SAPK/JNK signaling	26/100	0.000447
Hypoxia signaling in the cardiovascular system	20/64	0.000724
B cell receptor signaling	38/162	0.001349
Assembly of RNA polymerase III complex	7/14	0.001349
NRF2-mediated oxidative stress response	42/187	0.001413
NGF signaling	28/111	0.001905
Cdc42 signaling	31/143	0.001905
Pyridoxal 5'-phosphate salvage pathway	19/63	0.001950
Paxillin signaling	26/110	0.002570
Integrin signaling	44/205	0.003236
Pancreatic adenocarcinoma signaling	27/115	0.003981
Growth hormone signaling	19/71	0.005012
Melanocyte development and pigmentation signaling	22/87	0.005370
FLT3 signaling in hematopoietic progenitor cells	19/73	0.008318
CD40 signaling	17/68	0.0093325
Tartu vs. Petrozavodsk		
Ingenuity canonical pathway	Genes/pathway	P value
B cell receptor signaling	31/162	0.000001
FLT3 signaling in hematopoietic progenitor cells	16/73	0.000072
Acute myeloid leukemia signaling	16/81	0.000145
Fcγ receptor-mediated phagocytosis in macrophages and monocytes	18/95	0.000186
HMGB1 signaling	18/97	0.000214
p53 signaling	18/100	0.000417
Docosahexaenoic acid (DHA) signaling	10/41	0.000437
RAR activation	26/179	0.000646
Polyamine regulation in colon cancer	7/24	0.000933
NGF signaling	18/111	0.001096
HIF1α signaling	17/102	0.001479
IL-22 signaling	7/24	0.001660
IL-15 signaling	12/65	0.001995
Rac signaling	17/117	0.002042
AMPK signaling	20/143	0.002089
Espoo vs. Tartu		
Ingenuity canonical pathway	Genes/pathway	P value
Granzyme A signaling	3/17	0.000191
Renal cell carcinoma signaling	4/17	0.001148
FGF signaling	4/87	0.002570
Actin cytoskeleton signaling	6/230	0.002884
SAPK/JNK signaling	4/100	0.002951
Chronic myeloid leukemia signaling	4/102	0.003467
Telomerase signaling	4/99	0.004074
NGF signaling	4/111	0.005754
ErbB2-ErbB3 signaling	3/58	0.006026
Role of NANOG in mammalian embryonic stem cell pluripotency	4/113	0.006310
Insulin receptor signaling	4/131	0.010471
Neurotrophin/TRK signaling	3/70	0.010471
GDNF family ligand-receptor interactions	3/70	0.010965
Prolactin signaling	3/77	0.013183
PDGF signaling	3/79	0.015136

type 1 diabetes risk were analyzed with a lanthanide labeled oligonucleotide hybridization method as described previously [15]. The subjects were divided into four groups based on their HLA-associated risk for T1D [14] (Tables 1 and S1). The study protocols were approved by the ethical committees of the participating hospitals and the parents gave their written informed consent.

2.2. RNA isolation

Total whole-blood RNA was extracted from the samples using Tempus Spin RNA isolation kit (Applied Biosystems) according to manufacturer's instructions. RNA quality and quantity was determined using NanoDrop ND-2000 (Thermo Scientific) and Experion Automated Electrophoresis System (Bio-Rad Laboratories).

2.3. Array hybridizations

50 ng of total RNA was processed to cDNA with Ovation RNA amplification system v2, including the Ovation whole blood reagent (NuGEN Technologies). The amplified cDNA was subsequently biotin-labeled and fragmented with Encore biotin module (NuGEN Technologies). Samples were hybridized to whole genome GeneChip Human Genome U219 array plates (Affymetrix) in accordance with the manufacturer's protocols for using the GeneTitan Hybridization, Wash and Stain Kit for 3′ IVT Array Plates (Affymetrix). A GeneTitan MC Instrument (Affymetrix) was used to hybridize, wash, stain, and scan the arrays. The probe cell intensity data was summarized with Affymetrix GeneChip Command Consol 3.1. The microarray data discussed in this publication are accessible through GEO SuperSeries accession number GSE53473 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53473).

2.4. Microarray data processing and statistical analysis

The intensity data was pre-processed using robust multi-array averaging (RMA) [16]. The absent call filtering was performed by determining the threshold value empirically for each sample, as described by [17]. A two-component Gaussian Mixture model was fitted for the non-control probe sets of each chip with an Expectation Maximization algorithm, which was implemented using the mixtools-package in R [18]. The assumption is that each component of the distribution would correspond to a different source of signal (background and true expression). A probe set was filtered out if it was absent in at least 50% of the samples from Espoo, Tartu and Petrozavodsk.

As the Espoo and Tartu RNA samples were hybridized to array plates in December 2011–January 2012 and the Petrozavodsk samples in March 2013, the differences between Petrozavodsk and the other areas were confounded by a batch effect. In order to correct for this effect, 15 samples (seven from Petrozavodsk, four from Espoo and four from Tartu) were re-hybridized on the same Affymetrix U219 platform. The batch correction was made by applying the ComBat analysis method [19], which implements an empirical Bayes framework to adjust for the batch effect.

Differential expression was detected by using the R Bioconductor [20] package Limma [21] to fit a linear model and compute a moderated t-statistic for each present probe set for all three comparisons: Espoo vs. Petrozavodsk, Tartu vs. Petrozavodsk and Espoo vs. Tartu. Gender, pregnancy week, month of birth, and HLA risk class were included as confounding factors in the model. A probe set was deemed differentially expressed if its Benjamini–Hochberg-corrected P value, estimated as the false discovery rate (FDR), was lower than 0.01. The probe sets meeting this criteria are listed in Table S2.

2.5. Molecular pathway enrichment

Molecular pathway enrichment was tested using the Ingenuity Pathway Analysis (Ingenuity Systems, www.ingenuity.com) core analysis tool. The top 15 enriched canonical pathways among the differentially regulated genes are listed in Table 2.

2.6. Differentially expressed genes with functions related to the innate immunity responses

The human genes with literature-annotated function in the innate immunity were downloaded from www.innatedb.org [22]. The enrichment of these genes among our differentially expressed genes was calculated using Fisher's exact test and the Benjamini–Hochberg-corrected P values are reported in Table S3. Specific information on the function in innate immunity is listed beside each gene in Table S2 (InnateDB columns).

2.7. Differentially regulated genes associated with developmental regulation of the immune response

Two recent studies reporting transcriptomic changes in 1-year-old infants and newborn infants were selected for comparisons with our data. Martino et al. [23] studied LPS induction in mononuclear cells at birth and at 1 year of age. They reported 549 genes that were differentially expressed between these age groups. The enrichment of these genes among our differentially expressed genes was calculated using Fisher's exact test and the Benjamini–Hochberg-corrected P values are reported in Table S3 and Fig. 4.

Wynn et al. [24] performed whole blood transcriptomics upon bacterial sepsis infection in different age groups of children relative to healthy control children. From the associated pre-processed and normalized microarray data sets from Gene Expression Omnibus (GSE26440 and GSE26378), data from neonates (age < 1 month) and infants (0.5 years < age < 1.5 years) was selected. In both age groups, approximately 1/3 of these samples were from healthy controls and 2/3 were from patients with bacterial sepsis. After fitting a linear model, a moderated t-statistic [21] was computed for each probe set, taking health status (sepsis/healthy) and survival as confounding factors in the analysis. There were 2205 genes that were identified as upregulated and 1432 genes downregulated in the older infants compared to the neonates. The enrichment of these genes among our differentially expressed genes was calculated using Fisher's exact test. The Benjamini–Hochberg-corrected P values are reported in Table S3 and Fig. 4.

3. Results

3.1. Place of birth affects hundreds of genes in the umbilical cord blood transcriptome

Using the criteria of an adjusted P value < 0.01, 3442 probe sets were differentially regulated between Espoo and Petrozavodsk, 130 probe sets between Espoo and Tartu, and 1655 probe sets between Tartu and Petrozavodsk (Fig. 2, Table S2). A majority of the differences were observed between Espoo and Petrozavodsk. The umbilical cord blood transcriptome of infants born in Tartu shared greater similarity with the infants born in Espoo, than to that of infants born in Petrozavodsk.

Molecular pathway enrichment was tested by using the IPA core analysis pathway tool (Table 2). Interestingly, the B cell receptor signaling pathway was significantly enriched among the differentially expressed genes in samples from Espoo and Tartu compared to Petrozavodsk. For example, *CD79B* coding for B cell antigen receptor Ig-beta protein, and *PAX5* coding for transcription factor crucial for B cell lineage commitment, were suppressed in samples from Petrozavodsk. Differences in the NGF and CD40 signaling pathways were mainly due to the downregulation of MAPK signaling molecules in Espoo and Tartu. Pathways linked to FLT3 activity (FLT3 signaling in hematopoietic stem cells and acute myeloid leukemia signaling) were suppressed in samples from Espoo and Tartu relative to samples from Petrozavodsk. FLT3 is a plasma membrane receptor expressed by hematopoietic stem and progenitor cells in the bone marrow, thymus and lymph nodes, and is required for example for the normal proliferation and development of dendritic cells (DC) in the periphery [25].

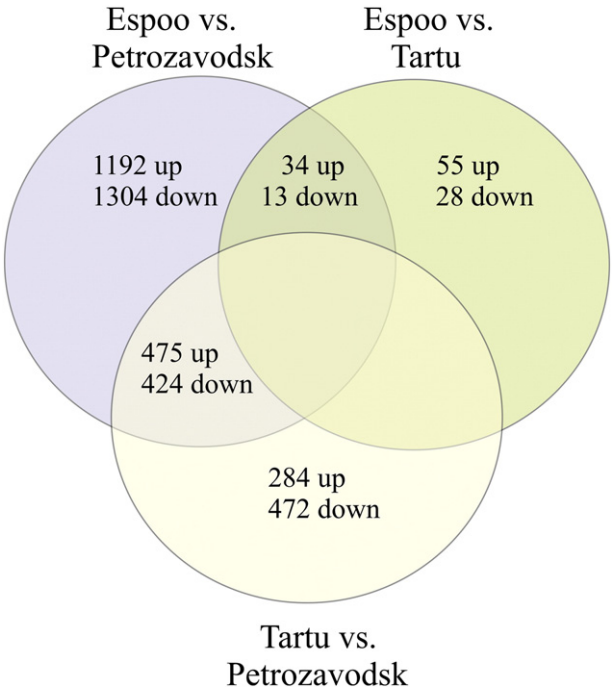

Figure 2 Summary of the number of probe sets showing differential regulation (corrected P value < 0.01, moderated t-test) between the cord blood transcriptome of infants born in Espoo (Finland, n = 48), Tartu (Estonia, n = 25) and Petrozavodsk (Russian Karelia, n = 40).

3.2. Common transcriptome regulation in Finnish and Estonian children when compared to Russian Karelia

From the comparisons with the Petrozavodsk samples, the Espoo and Tartu infants shared four hundred seventy-five upregulated probe sets and 424 downregulated probe sets (Fig. 2, Table S2). As a highlight, the ZFP36 ring finger protein-like 1 (*ZFP36L1*) gene was highly upregulated both in Espoo and Tartu when compared to Petrozavodsk (2 and 1.8-fold, Fig. 3A). ZFP36L1 is a RNA binding protein involved in a variety of mRNA processing steps that is expressed in lymphoid and myeloid hematopoietic lineages, and inhibits erythroid differentiation through degradation of Stat5b mRNA [26]. ZFP36L1 has also been shown to maintain the immature state of B cells and negatively regulate plasma cell differentiation by targeting transcription factor BLIMP1 [27]. Notably, SNPs in the proximity of this gene reside in a genetic locus shared between many autoimmune diseases, and these SNPs have been associated as lead SNPs with celiac disease (CD), Crohn's disease and type 1 diabetes [28]. *PTCRA* was also upregulated in children from both in Espoo and Tartu, relative to Petrozavodsk (1.8 and 1.7-fold, Fig. 3B). *PTCRA* codes for pre-T cell antigen receptor alpha, which together with T cell receptor β forms a pre-TCR complex in immature T cells. This receptor then facilitates the expansion and differentiation of precursor T cells. PTCRA expression has been observed to increase in the double negative (CD4- CD8-) stage of the αβT cell development, and then gradually decline when cells reach the single positive stage [29].

3.3. Differentially regulated genes associated with innate immune responses

Newborn infants are mostly dependent on their innate immunity to fight against pathogens. In order to test whether the detected expression differences were related to the functions of the innate immune system, the differentially regulated genes were compared against the InnateDB [22]. Genes of the innate immune system were enriched in the upregulated genes in Petrozavodsk, when compared to Espoo and/or Tartu, and in the downregulated genes in Petrozavodsk, when compared to Espoo (Table S2, Innate Immunity columns, and Table S3). The innate immunity genes upregulated in Petrozavodsk relative to Espoo and/or Tartu included several pattern recognition receptors responsible for microbial sensing, such as Toll-like receptor 2 (*TLR2*, 1.3-fold when compared to Espoo), an essential receptor in the defense against Gram-negative bacteria by the monocytes and macrophages, as well as absent in melanoma 2 (*AIM2*, 1.5 and 1.8-fold) that codes for the central cytosolic double-stranded DNA sensor of bacteria and DNA viruses [30]. In addition, Nod-like receptors C4 and C5 (NLRC4, NLRC5) were upregulated in Petrozavodsk when compared to Espoo (both 1.3-fold).

Complement component 4 binding protein alpha (*C4BPA*) was among the most highly upregulated genes in Petrozavodsk when compared to Espoo (2.4-fold) and Tartu (2.3-fold).

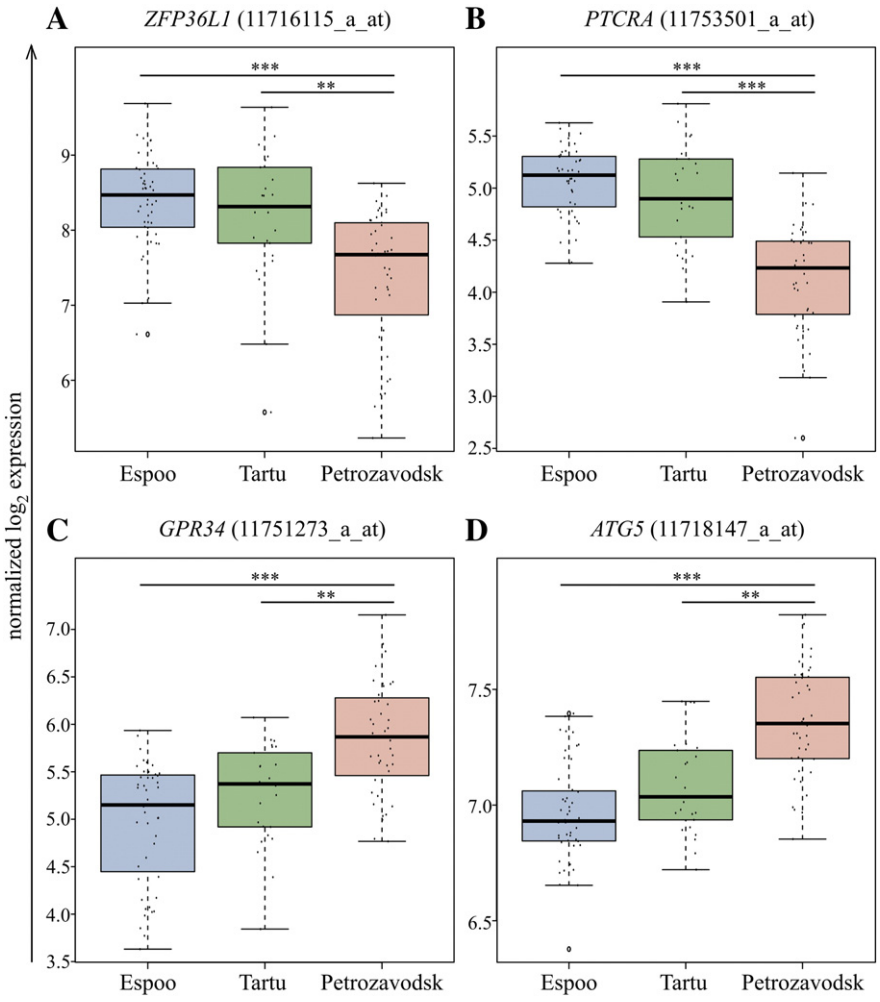

Figure 3 Example box plots of genes showing differential regulation in Petrozavodsk compared to Espoo and Tartu. (A) *ZFP36L1* coding for ZFP36 ring finger protein-like 1, (B) *PTCRA* coding for pre-T cell receptor alpha, (C) *GPR34* coding for G-protein coupled receptor 34, and, and (D) *ATG5* coding for autophagy related 5. Dark horizontal line represents the median, with the box representing the 25% and 75% percentiles. The whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box. The outliers are presented as open circles. **FDR < .01, ***FDR < .001 (moderated t-test).

Together with C4BPB (beta isoform) it can form the C4BP complex (with $\alpha7\beta1$, $\alpha6\beta1$, and $\alpha7\beta0$ compositions) and act as the major inhibitor of the classical and lectin complement pathways. Additionally, it has been observed to activate B-cell proliferation and IgE production through binding to CD40 [31], and more recently the $\alpha7\beta0$ isoform was shown to induce a tolerogenic, anti-inflammatory state in DCs [32]. Another complement inhibitor *CD46* (also known as membrane cofactor protein, MCP) was also upregulated in the samples from Petrozavodsk (1.4-fold in both comparisons). This transmembrane protein is ubiquitously expressed to protect autologous cells from complement activation by binding to C3b and C4b complement proteins and promoting their degradation. Similarly to C4BPA, CD46 has diverse functions beyond the complement system: CD46 activation has been reported to be crucial for type 1 interferon production in macrophages [33]. CD46 also promotes T regulatory cell (Treg) induction towards the IL-10 secreting anti-inflammatory Type 1 Treg (Tr1) subset [34], and even from polarized T helper type 1 (Th1) cells [35]. In addition to C3b and C4b complement proteins, CD46 also binds to Jagged1, which prevents the interaction of Notch1 and Jagged1 in resting T cells and prevents IFNγ production [36].

3.4. Differentially regulated genes associated with developmental regulation of the immune response

With a view to establish whether these contrasting expression levels were related to differences in the developmental or maturation status of the neonatal immune system between the study sites, we compared our observations with two recent studies that reported transcriptomic changes occurring in monocytes by LPS induction at birth and at 1 year of age [23] and in whole blood upon bacterial sepsis infection between different age groups of children relative to healthy control children [24]. Both studies reported profound differences in neonatal responses compared to the other age group(s). The analyses revealed that genes upregulated in Petrozavodsk (downregulated in Tartu and/or Espoo) had a significant overlap with the LPS induced genes upregulated at 1 year of age compared to newborn infants (Table S3). From our

re-analysis of the public of the sepsis study [24], we identified genes that were up- and downregulated between neonates and 1-year-old children. In similarity to the LPS dataset, genes upregulated in Petrozavodsk (downregulated in Tartu and/or Espoo) had a significant overlap with genes upregulated in sepsis at 1 year of age (Table S3). Genes upregulated in Espoo and/or Tartu relative to Petrozavodsk were found to overlap with the upregulated genes associated with sepsis in neonates. P values for the enrichment for developmentally regulated immune system transcripts are represented in Fig. 4.

One of the genes upregulated in Petrozavodsk, with LPS at 1 year of age, and in 1-year-old sepsis patients was prolylcarboxypeptidase (*PRCP*, 1.2-fold compared to Espoo). *PRCP* encodes for a plasma membrane enzyme responsible for activation of kallikrein that subsequently leads to the production of vasodilator bradykinin (BK), both of which are components of the so called plasma contact system. PRCP expression and bradykinin production have been observed to be induced by LPS stimulation [37] and the contact system has recently been identified as one arm of the innate immune system due to its activities in inhibiting bacterial growth [38]. G-protein coupled receptor 34 (GPR34) was among the genes upregulated in newborn infants from Petrozavodsk and with LPS at 1 year of age (1.8 and 1.5-fold when compared to Espoo and Tartu, Fig. 3C). GPR34 is highly expressed on mast cells and less abundantly also in other immune cell types [39]. The functional significance of this receptor remains unclear, but it was recently associated with controlling T-cell responses and pathogen clearance, as knockout mice exhibited increased paw swelling in the delayed type hypersensitivity test and were also more susceptible to the fungal pathogen *Cryptococcus neoformans*. Among the genes associated with sepsis at 1 year of age and upregulated in Petrozavodsk, autophagy related 5 (*ATG5*, 1.3 and 1.2-fold, Fig. 3D) has been widely connected to immune functions, such as recognition of single-stranded RNA viruses and type 1 interferon production in DCs [40], antigen presentation on DCs and subsequent CD4+ T-cell priming [41], as well as to immune mediated diseases, such as multiple sclerosis [42] and asthma [43].

4. Discussion

This study has investigated the influence of the environment, modern vs. traditional differing in the incidence of both infectious and immune-mediated diseases, on the cord blood transcriptome at birth. Although no major differences were detected between samples from Espoo (modern society) and Tartu (rapidly developing society), samples from Petrozavodsk (traditional society) differed from the samples taken from infants born in other study sites, and especially from Espoo. Upregulation of several pivotal innate immune

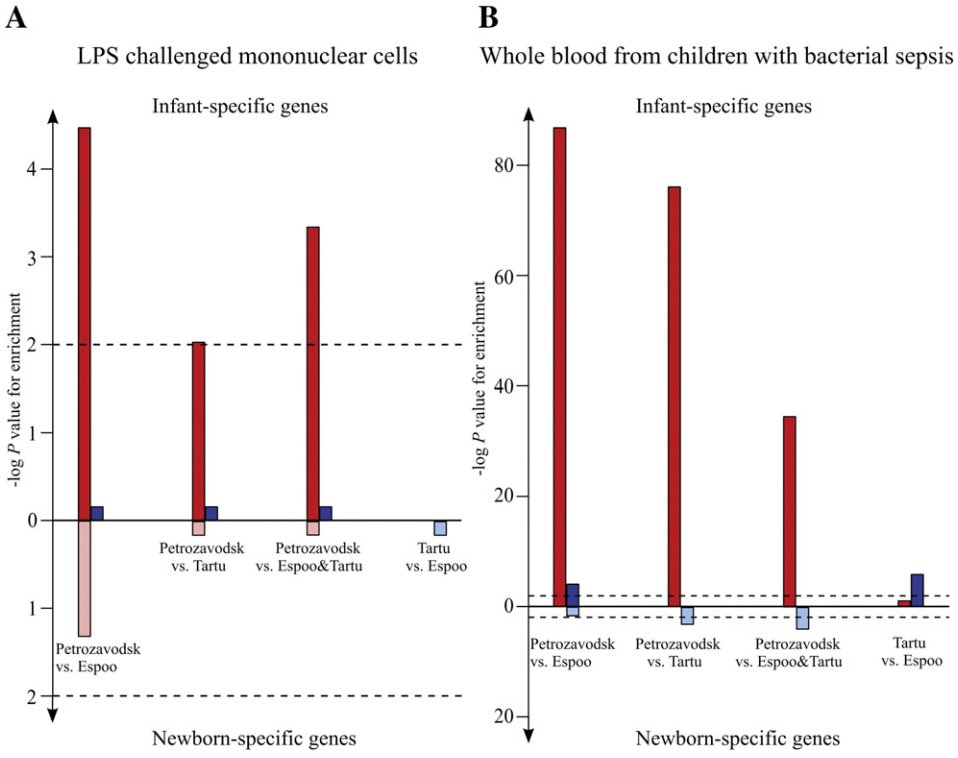

Figure 4 Developmental regulation of the immune response in cord blood in the three study sites. Logarithmic Benjamini–Hochberg corrected P values for enrichment (Fisher's exact test) of the differentially regulated genes with genes differentially regulated by (A) LPS in mononuclear cells isolated from newborn infants or 1-year-old infants [23] or (B) bacterial sepsis in whole blood of newborn infants or 1 year-old infants [24] are presented. Enrichment overlap with infant-specific genes is presented on the upper panel, and with newborn-specific genes on the lower panel. The red bars correspond to upregulated and blue bars to downregulated genes. The cutoff for enrichment significance (P = 0.01) is presented with dashed horizontal lines. See Table S3 for further details on the overlapping genes and P values.

response mediators, such as *TLR2*, *AIM2*, *ATG5*, and *IRF7* in samples from Petrozavodsk was evident. Interestingly, TLR2 has been reported to be upregulated in mononuclear cells at birth upon infection with Gram-positive bacteria [44], and conversely downregulated in cord blood CD34+ progenitor cells of infants at high-atopic risk [45]. IRF7 on the other hand plays a central role in the viral defense and in the induction of the interferon response [46], and shows genetic association with susceptibility to type 1 diabetes [47]. It has also been suggested that the innate immune responses play a role in the development of allergy. For example, a multinational study demonstrated an association between maternal contact with farm animals during pregnancy, higher expression of CD14 and toll-like receptors 1 to 9, and a protective effect on the development of atopic dermatitis in the first 2 years of life [48]. Therefore, the detected activation of innate immunity genes in Petrozavodsk newborn infants, which is usually associated with subsequent Th1 responses, might confer later protection from aberrant Th2 responses, associated with atopy and asthma.

Notably, genes that were upregulated in samples from Petrozavodsk relative to those from either Espoo or Tartu overlapped with genes previously identified to be induced by LPS in mononuclear cells at 1 year of age, but not at birth. The upregulated signature in samples from Petrozavodsk also overlapped with that of 1-year-old children with bacterial sepsis (Fig. 4). Altogether this suggests that the infants born in Petrozavodsk have been exposed to TLR ligands in utero, and that the subsequent immune response is developmentally more advanced, resembling that of matured infants. In support of this, it was recently reported that neonatal antigen presenting cells (APC) from Papua New Guinean (traditional environment) had a more activated and mature baseline status than APCs from Australian (modern environment) neonates, as determined by increased expression of activation markers HLA-DR and CD86, but that the APCs from Papua New Guinea were also more quiescent and resistant to further LPS stimulation [49]. This supports the implication that the surrounding bacterial environment is preprogramming the immune responses in utero, and that this environment would differ in Petrozavodsk versus the other study sites. In fact, data on house dust sample analysis in Finnish and Russian Karelian homes revealed striking differences, as both the amount of bacterial content and diversity were higher in Russian Karelia than in Finland [50]. Lisciandro et al. [49] suggested that the differences in the state of activation of antigen presenting cells from neonates between modern and traditional environments reflect an evolutionary mechanism of immune regulation learned in utero to prepare newborn infants for the intensity and frequency of the immunological challenges in the postnatal environment and to protect them from infections.

Markers associated with immature immune cell phenotypes, such as *ZFP36L1* inhibiting plasma cell and erythroid differentiation, and pre-T cell receptor alpha (*PTCRA*) expressed in immature T cells, were upregulated in newborn infants from Espoo and Tartu (Figs. 3A–B). ZFP36L1 downregulation, similar to that seen in samples from Petrozavodsk, has been observed in murine B cells after LPS stimulation and during the shift from mature B cell to plasma cell [27]. This suggests that not only innate immune cells, but also adaptive T and B cells in newborn infants from Espoo and Tartu have a more immature phenotype and have not been activated and/or differentiated to the extent observed in Petrozavodsk infants. On the other hand, it also appeared that inhibitory or homeostasis mechanisms were activated in Petrozavodsk, such as CD46 with a role of suppressing Th1 responses in infection [36]. CD46-induced Tregs have been hypothesized to ensure unresponsiveness to commensal bacteria while maintaining defense against invading pathogens by allowing DC maturation [51]. Interestingly, CD46 responsiveness is defective in T cells from patients with multiple sclerosis [52], asthma [53] and juvenile and rheumatoid arthritis [35]. In this respect, upregulation of the multifunctional C4BPA in Petrozavodsk was an interesting finding. This protein has immunomodulatory downstream effects on DCs, as was demonstrated when treatment of DCs with LPS and C4BPA promoted Treg differentiation instead of inducing activation markers and Th1 programming [32]. It is intriguing to speculate, that such a tolerant state of the APCs and lymphocytes induced in utero would lead to different immune responses upon colonization with the commensal flora or infections after birth, and that a similar protective shift in the prenatal host-microbe interaction might be achieved by probiotics in the modern environments [54].

As this study was performed using umbilical cord whole blood samples consisting of heterogeneous cell populations, it was not possible to identify which cell types were responsible for the detected differences. This warrants further investigation and validation, for example the frequency of different cell populations needs to be analyzed more carefully. In addition, a number of differentially expressed cell surface proteins, such as CD46 and FLT3, could be quantified simultaneously. An unavoidable limitation of this study was the collection of samples at three different study sites, although possible batch effects were kept at minimum by stabilizing the RNA immediately after the blood draw into the preservative buffer present in the collection tubes, as well as storing the samples at −70 °C until processing and analysis in the same laboratory. Any batch effects related to hybridization were eliminated during the data analysis as well as possible. In the light of the differences observed within these data, repetition and validation of the study in another cohort of neonates born in contrasting standards of living should strengthen this hypothesis. Moreover, the influence of maternal diet, microbiome, infections, and the course of pregnancy and child birth would certainly be of interest for future investigations. Additionally, genetic differences between the populations could partly explain the results, and therefore the T1D-related HLA risk class was regarded as one of the confounding factors in the analysis. However, based on the genome-wide SNP genotyping, Estonians and Russians are rather similar to each other, differing clearly from the Finns [55]. Therefore, the genetic factors are an unlikely explanation for the similarities detected here between Finland and Estonia, or for the dissimilarities detected between Estonia and Russia.

In summary, we report here the first whole-genome transcriptomic study describing the differences in immunologically active genes in the umbilical cord whole blood samples from neonates born in contrasting standards of hygiene and living. The findings support the hygiene hypothesis, but at the same time extend the immunomodulatory window to the prenatal period. The environmental factors that play a role in the susceptibility/protection towards immune-mediated diseases begin to shape the neonatal immunity already in utero

and direct the maturation of both the adaptive and the innate immune responses in accordance with the surrounding microbial milieu. This could then have far reaching effects on immune functions later in childhood, and dictate the outcome of the balance between efficient immune responses against pathogens and development of immune mediated diseases.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2014.09.009.

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